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The Role of Epigenetic Modifications in Neurodevelopmental Disorders: A Systematic Review

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Highlights

- Epigenetic modifications play a role in neurodevelopment.
- Few epigenetic markers have been repeatedly identified in relation to ASD or ADHD.
- Suboptimal designs and heterogeneity hamper the interpretation of findings.
- Large hypothesis-free studies can provide insight into the epigenetics of ASD/ADHD.

ABSTRACT

Epigenetic processes have been suggested as key mechanisms in the etiology of neurodevelopmental disorders. This systematic review summarizes the current evidence for an association between epigenetics and Autism Spectrum Disorder (ASD) and Attention/Deficit-Hyperactivity Disorder (ADHD). Six databases were searched until the 24th of October 2017. Of the 2169 retrieved articles, 29 met our inclusion criteria. While generally associations between epigenetics and neurodevelopmental disorders were reported, only a few findings were consistent across independent analyses. Differential epigenetic markers were repeatedly identified in *OR2L13, C11orf21/TSPAN32, PRRT1* and H3K27 for autism, and in *VIPR2* for ADHD. Overall, evidence of an association between epigenetic modifications and ASD or ADHD should be considered preliminary and based on studies suffering from numerous caveats. We highlight the need for carefully designed investigations and for greater homogeneity and provide specific recommendations for future research. Despite our current limited understanding, the suggestive findings and the rapid advances in the field hold the promise of a forthcoming elucidation of the role of epigenetic modifications in neurodevelopmental disorders.

Key Words: ASD; ADHD; DNA methylation; histone modifications; epigenetics.

1. INTRODUCTION

Neurodevelopmental disorders are psychiatric conditions arising in early life featuring abnormalities in the central nervous system development. These can lead to emotional and behavioral problems and impairments in psychological, social, academic and occupational functioning (American Psychiatric Association, 2013). Autism Spectrum Disorder (ASD) and Attention-Deficit/Hyperactivity Disorder (ADHD) are among the most common and well-known neurodevelopmental conditions, afflicting about 1% and 5% of children, respectively (American Psychiatric Association, 2013). A rise in the prevalence rates of these conditions has recently been reported (American Psychiatric Association, 2013; Boyle et al., 2011), highlighting the need for careful explorations of pathways to disorder etiology; although higher rates might result from confounding factors such as increased disorder awareness and methodological issues (American Psychiatric Association, 2013; Polanczyk et al., 2014).

ASD is characterized by restricted and repetitive behaviors and impairments in social interaction and communication. Autism is currently conceptualized as a *spectrum* of disorders capturing the commonalities across previously identified subtypes (e.g. Asperger's Disorder, Autistic Disorder) while allowing for heterogeneity within this condition (American Psychiatric Association, 2013). For instance, individuals requiring very substantial support as well as highly functioning persons can meet the criteria for this disorder. ADHD is defined by hyperactivity, impulsivity, inattention and difficulties in organization atypical for the age of the child. Individuals suffering from ADHD can exhibit clinical hyperactivity/impulsivity and inattention (combined presentation) or the predominance of either of these symptoms (predominantly inattentive presentation, predominantly hyperactive/impulsive presentation) (American Psychiatric Association, 2013).

Both neurodevelopmental disorders tend to persist into adulthood and can often co-occur, with the manifestation of ADHD symptoms in 30% to 50% of autistic patients (Leyfer et al., 2006; Lichtenstein et

al., 2010; Taurines et al., 2012). The etiology of neurodevelopmental disorders is complex and involves multiple determinants. A substantial genetic component is present in both psychiatric conditions (American Psychiatric Association, 2013; Elia et al., 2012; Faraone et al., 2005; Loke et al., 2015; Tordjman et al., 2014). Furthermore, environmental factors, such as prenatal maternal stress and exposure to toxins, play a considerable role (American Psychiatric Association, 2013; Kubota and Mochizuki, 2016; Loke et al., 2015; Schuch et al., 2015); however, the processes through which the environment, by interacting with genetic susceptibilities, leads to the development of neurodevelopmental disorders are yet to be elucidated (Keil and Lein, 2016).

Epigenetic modifications have been suggested as the mechanisms responsible for the biological encoding of environmental influences, representing the meeting point of genes and environment (Feil and Fraga, 2012; Keil and Lein, 2016; LaSalle, 2013; Latham et al., 2012). Epigenetic processes can regulate gene expression, without affecting underlying DNA sequences (Feil and Fraga, 2012). While there is growing evidence that epigenetic processes are sensitive to numerous environmental exposures (e.g. nutritional factors) (Feil and Fraga, 2012), the effect of genetic variation on epigenetic markers has also been documented (Kilpinen and Dermitzakis, 2012). Regardless of their determinant, epigenetic modifications are considered relevant factors in the etiology of complex disorders such as ASD and ADHD, which could account for the unexplained variance of disorder occurrence.

The most studied epigenetic changes are DNA methylation and histone modifications. The former occurs when a methyl group is added to specific DNA base pairs, primarily in the context of cytosine-guanine dinucleotides (CpGs). Methyl groups regulate gene expression determining activation or inhibition of activity, according to the methylated location (e.g. gene body, CpG island) (Moore et al., 2013) and to the degree of DNA methylation, typically expressed as hypomethylation (i.e. lower levels of DNA methylation) or hypermethylation (i.e. higher levels of DNA methylation). The three most common approaches to profile DNA methylation patterns are global, epigenome-wide and candidate gene DNA methylation analyses. Global DNA methylation provides an average estimate of DNA methylation levels

in the analyzed tissue sample by quantifying the methylcytosine (5-mC) present in the genome (Non and Thayer, 2015). In contrast, candidate gene DNA methylation studies and Epigenome-Wide Association Studies (EWAS) examine DNA methylation at specific CpG sites or regions and are thus defined as site-specific studies. More precisely, EWAS are typically hypothesis-free and screen up to hundreds of thousands of loci across the genome to identify CpGs or regions associated with the disorder of interest. In contrast, candidate gene DNA methylation analyses target loci in one or a limited number of specific genes, based on *a priori* hypotheses.

Post-translational histone modifications are a group of epigenetic alterations (e.g. lysine methylation and histone acetylation) which remodel chromatin structure and which are associated with variations in DNA expression (Bannister and Kouzarides, 2011). Histone acetylation is consistently related to transcriptional activation (Kouzarides, 2007), whereas the transcriptional consequences of methylation are determined by the position of such modification. For instance, while methylation in histone three lysine four (H3K4) is involved in activation of DNA expression, methylation in histone three lysine 27 (H3K27) is implicated in repression.

Multiple publications have provided evidence of differential DNA methylation between cases and controls in ASD and ADHD (Loke et al., 2015; Walton et al., 2017) and of histone modifications in patients with ASD only (Sun et al., 2016). However, a comprehensive assessment of the epigenetic modifications present in both neurodevelopmental disorders is currently lacking. Therefore, the aim of this systematic review is to identify, integrate and discuss all available evidence on the association of DNA methylation and histone modifications with ASD and ADHD.

2. MATERIALS AND METHODS

2.1 Literature Search

In order to conduct this review, we followed a predefined protocol adhering to the PRISMA and MOOSE guidelines (Appendices 1 and 2). The search of six bibliographic databases (Embase.com, Web-of-

Science, Medline (Ovid), Cochrane Central, PsycINFO Ovid and Google Scholar) was performed on January 12th 2017 and updated on October 24th 2017 (last data search), in collaboration with a medical information specialist. The searched terms referred to both exposure (e.g. epigenetics, histone, DNA methylation, CpG) and outcomes (e.g. neurodevelopmental disorders, ASD, ADHD). We limited the search to studies on humans, but no language, tissue type (*in vivo* or *post-mortem*) or publication date restrictions were applied. The complete search strategies can be found in Appendix 3. To retrieve further relevant publications, we consulted experts and examined literature reviews on the topic.

2.2 Study Selection and Inclusion Criteria

Studies were included if they described the association between epigenetic marks (DNA methylation or histone modifications) and ASD or ADHD. The excluded studies *(i)* referred to epigenetic marks different from histone modifications and DNA methylation; *(ii)* examined neurodevelopmental disorders other than ASD and ADHD, such as Down Syndrome, Fetal Alcohol Spectrum Disorder and Intellectual Disability; *(iii)* were not empirical studies; *(iv)* did not compare cases to controls. Psychiatric disorders other than ASD and ADHD were excluded in order to limit the scope of our systematic review to two of the most common and comorbid forms of neurodevelopmental conditions. Additionally, the traits (e.g. high negative affect) and etiological factors shared by ASD and ADHD (Visser et al., 2016) point to the possibility of similar biological profiles. Other disorders of neurodevelopmental etiology, such as schizophrenia, have been reviewed elsewhere (Fullard et al., 2016; Homberg et al., 2016; van Bokhoven, 2011). Two independent reviewers selected the publications meeting the inclusion criteria by examining titles and abstracts. In case of disagreement a senior researcher was available, but all divergences regarding the chosen articles were discussed and solved between the two reviewers. Full texts of the studies of interest were retrieved and a further selection was performed according to the inclusion and exclusion criteria.

2.3 Data Extraction

The relevant information from the studies was collected in predesigned data forms, which varied according to the type of approach (global, candidate gene, epigenome-wide DNA methylation and histone modifications) and which were subdivided by tissue examined (brain tissue or peripheral – other tissues such as blood and saliva-). These data forms included the neurodevelopmental disorder outcome (ASD or ADHD), sample size (cases/controls), sample characteristics (population, age range or mean age and sex), tissue type, the methodology for epigenetic marks assessment (type of statistical analysis and platform), the presence of validation or replication, adjustments or matching and a summary of the findings with reported statistics (e.g. *p*-values) (Tables 1-6). Of note, validation refers to the verification of findings in the same sample with the use of a different methodology. Replication instead entails testing a new group of participants.

When the required information was not presented in the included articles, we contacted correspondence authors. If there was no response, we classified the missing information as "not available" (NA). Data extraction was performed by one author, who was assisted by a research analyst in the identification of platforms used.

2.4 Assessing the Risk of Bias

Although PRISMA and MOOSE guidelines require quality assessment of the included studies, we decided not to perform a formal evaluation due to the lack of specific guidelines for epigenetic studies (e.g. required sample size according to approach, platform coverage, performance of validation).

2.5 Outcome Assessment and Statistical Methods

For each study, we defined whether an association was present and, when applicable, effect sizes were reported. We sought to pool the results using a random effects meta-analysis model. However, due to high heterogeneity in the input parameters, assumptions and study design, it was not feasible to perform quantitative pooling of the existing data.

3. RESULTS

As shown in Figure 1, through a literature search and other sources, we identified 2169 potentially relevant articles. Of these, 29 met the eligibility criteria and were thus included in this review. Reasons for exclusion of individual articles can be disclosed upon request. In the following section, we provide a summary of all included studies, followed by a review of their findings. Results are presented for each neurodevelopmental disorder separately and further subdivided according to the approach employed (global DNA methylation, candidate gene DNA methylation studies, EWAS and histone modifications) and tissue examined (brain, peripheral).

3.1 Summary of included studies

Overall, ASD was examined in 24 publications (global DNA methylation, candidate genes, EWAS and histone modifications) and ADHD in five (candidate genes and EWAS). No study focused on epigenetic marks in both disorders.

Cross-sectional designs comparing cases and controls were commonly used (*n*=24). A few publications examined participants prospectively in nested case-control studies (Schroeder et al., 2016; Van Mil et al., 2014; Walton et al., 2017) or compared monozygotic twins discordant for the disorder of interest (Nguyen et al., 2010; Wong et al., 2014). Of the included studies, 11 sampled individuals in the USA, three in China and two in the UK. The remaining studies were from Spain, Croatia, Italy, The Netherlands, comprised multiple samples of participants from mixed countries, or, did not specify the sample nationality. The selected publications mainly included individuals with mixed age at epigenetic assessment (*n*=15). Other articles sampled the tissues of the participants during the neonatal period (*n*=3), childhood (*n*=5) or adolescence (*n*=1). Of these, Walton et al. (2017) assessed epigenetic marks at multiple time points during the neonatal period and childhood. No study targeted an adult population only, while six publications did not disclose the patients' age. Regarding tissue sampling, 17 studies analyzed peripheral tissue (blood, lymphoblastoid cell lines, leucocytes, cord blood, buccal epithelium, placenta, and saliva) while12 used *post-mortem* brain tissue (cerebellum, cortices - temporal, occipital,

(pre)frontal cortex -, pons, Brodmann's areas and fusiform gyrus). The most commonly examined tissues were blood and cerebellum.

The majority of the publications used a candidate gene (*n*=14) or EWAS (*n*=14) approach to test the presence of differential epigenetic marks. Few studies only quantified global DNA methylation (*n*=5) or histone modifications (*n*=2). Five publications employed multiple approaches to epigenetic analysis as shown in Supplementary Table S1. Different methods were used to assess global DNA methylation status (e.g. HPLC tandem mass spectrometry (LC-MS/MS)). The included site-specific methylation studies mainly examined differentially methylated positions (DMP) or differentially methylated regions (DMR). Most of the candidate gene DNA methylation studies used bisulfite pyrosequencing, a quantitative approach with high reproducibility, but with relatively short length of reads (Non and Thayer, 2015). The most commonly used platform in epigenome-wide analyses was the Illumina Infinium HumanMethylation450K BeadChip (Infinium (HM)450K array)), which allows for the screening of over 450'000 CpG sites with high quantitative accuracy (Non and Thayer, 2015). The platform covers approximately 2% of all human CpG sites and underrepresents several genomic components such as enhancer regions (Barker et al., 2017). Of the 14 EWAS, four included an independent sample to confirm the findings from the discovery analysis (Aldinger et al., 2013; Homs et al., 2016; Ladd-Acosta et al., 2014; Wilmot et al., 2016), while ten validated their results using an independent approach in the same cluster of participants (e.g. Berko et al., 2014; Nardone et al., 2017). Of the candidate gene DNA methylation studies, only one study conducted a validation analysis (Jiang et al., 2004). Adjustments or matching for age or sex were generally performed. A few studies controlled for additional covariates such as batch effects, cell heterogeneity, and post-mortem brain interval. More information on the characteristics of the included publications can be found in Tables 1-6.

3.2 Autism Spectrum Disorder

3.2.1 *Global DNA Methylation*

Global DNA methylation status of autistic patients and controls was compared in five publications, two focusing on brain and three on peripheral tissue. Characteristics and findings of global DNA methylation studies investigating ASD are available in Table 1.

Brain Tissue: No consistent associations between global DNA methylation and autism were identified by two independent studies in brain tissue. James et al. (2013) detected statistically significant DNA hypermethylation through Tandem Mass Spectrometry (LC/MS/MS) in the cerebellum of 13 autistic subjects when compared to 13 controls of mixed age ($M \pm SD$ = 5.9±1.2 in cases, 4.7±1.1 in controls). Contrasting results were reported by Mitchell et al. (2012), who did not find proof for significant global DNA methylation differences in LINE-1 elements in Brodmann's area 19 of 12 cases and 12 controls of mixed age.

Peripheral Tissue: No consistent associations of global DNA methylation with autism were reported by three independent studies in peripheral tissue. Two articles did not find evidence for differences in whole blood, placenta and cord blood of children up to 15 years by employing MethylC-sequencing and Luminometric Methylation Assay (LUMA) (Schroeder et al., 2016; Wong et al., 2014). Tsang et al. (2016), who used restriction enzymes for their research, found DNA hypermethylation in the leucocytes of 280 autistic five-year-old children ($\Delta\beta$ = 54.35±21.37).

3.2.2 *Epigenome-Wide DNA Methylation*

The association between genome-wide DNA methylation and ASD was examined in 12 studies, five focusing on brain and seven on peripheral tissue. Characteristics and findings of the included epigenome-wide analyses investigating ASD can be found in Table 2.

Brain Tissue: Associations between differential DNA methylation and autism in a genomic area encompassing Chromosome 11 open reading frame 21 (*C11orf21*) and Tetraspanin 32 (*TSPAN32*) were found by three independent analyses (Ladd-Acosta et al., 2014; Nardone et al., 2014). Ladd-Acosta et al. (2014) sampled the cerebellum and temporal and prefrontal cortices of 20 cases and 21 controls in a genome-wide significant regional change analysis. They reported DNA hypomethylation of

C11orf21/TSPAN32 (ΔM = -6.6%) in the temporal cortex and replicated their findings in different brain regions (cerebellum and pre-frontal cortex) within the same genomic region. Consistent with these results, Nardone et al. (2014) showed differential DNA methylation of *C11orf21/TSPAN32* in Brodmann's area 10 between 12 cases and 12 controls of mixed age and validated this finding ($\Delta\beta$ = 0.39 to -4.93). Associations in Proline Rich Transmembrane Protein 1 (*PRRT1*) were consistently identified by three analyses. Ladd-Acosta et al. (2014) reported DNA hypomethylation of regions in *PRRT1,* both in the discovery (ΔM = -7.8%) and replication samples. Nardone et al. (2014) additionally found differential DNA methylation in CpGs in *PRRT1*.

Other findings of differential DNA methylation were identified in sites/regions in, or near, *SNORD* family genes in frontal and temporal cortices and cerebellum (Feinberg et al., 2015), *ZFP57* (ΔM = 13.9%), *SDHAP3* (ΔM = 15.8%) in prefrontal and temporal cortices and cerebellum (Ladd-Acosta et al., 2014), *HDAC4, C1qA, IRF8* and *CTSZ* in Brodmann's areas 10 and 24 (Nardone et al., 2014), *GABBR1, ABAT, Mir124-1, Mir124-2, FAM124B, InNEAT1* in prefrontal cortex, Brodmann's areas 10 and 24 (Nardone et al., 2017). Of these, *C1qA* ($\Delta\beta$ = -3.45), *IRF8* ($\Delta\beta$ = -3.42) and *CTSZ* ($\Delta\beta$ = -2.13 to -3.39) (Nardone et al., 2014), *GABBR1* and *Mir124-2, FAM124B* and *InNEAT1* (Nardone et al., 2017) were validated. An epigenome-wide analysis of Brodmann's area 19, occipital and cerebellar cortices found no evidence for differences in DNA methylation between cases and controls of mixed age (Ginsberg et al., 2012).

Peripheral tissue: Associations between differential DNA methylation and autism in Olfactory Receptor family 2 subfamily L member 13 (*OR2L13*) – a gene involved in the neuronal response to odorants - were found by two independent studies sampling peripheral tissue (Berko et al., 2014; Wong et al., 2014). Berko et al. (2014) reported differential DNA methylation between 47 cases and 48 controls in buccal tissue in a cross-sectional study comprised of participants of mixed age (β = -8.0%). Wong et al. (2014), in a longitudinal analysis from birth till age 15, found differential DNA methylation of *OR2L13* between 66 cases and 44 controls in whole blood ($\Delta\beta$ = 0.18); this finding was validated. Differentially

methylated markers in *ENO2* – a gene coding for an isoenzyme present in mature neurons - were partially identified by two independent analyses carried out by Wang et al. (2014). In their discovery cohort, they showed DNA methylation differences in 228 gene promoters and 247 CpG islands, including the *ENO2* promoter region, between 5 autistic children and 5 controls ranging from three to twelve years in venous blood. In their replication sample, they confirmed hypermethylation in correspondence to the *ENO2* promoter in 15% of a sample comprising 131 pairs of cases and controls (*M* = 39.1% in cases, *M* = 18.81% in controls).

Other publications reported the presence of contrasting epigenetic patterns between cases and controls in or near the following genes: *TAC1, PTCD2, HOXA11, TCN1, ETNK2, SPAG7, FLJ44881* ($|\Delta\beta| > 0.15$) (Aldinger et al., 2013), *BCL2* (*M* = 25% in cases, *M* = 9.5% in controls) and *RORA* in lymphoblastoid cell lines (Nguyen et al., 2010), *DLL1* and *LOC15444* in placenta (Schroeder et al., 2016), *PIK3C3* ($\Delta\beta$ = -0.04), *NFYC, C14orf152* ($\Delta\beta$ = -0.16) and *MGC3207* ($\Delta\beta$ = -0.24) in blood (Wong et al., 2014), *PAX8* (β = -7.5%), *GPC1* (β = -8.6%), *ADRA2C* (β = -11.9%), *FAM134B* (β = -7.8%), *CREB5* (β = 7.6%), *NOS1* (β = 8.6%), *MAPK8IP3* (β = 9%), *HOOK2* (β = -7.9%), *NRG2* (β = 7.6%), *KCNQ5* (β = 7.7%), *ZG16B* (β = 10.1%), *LOC643802* (β = -8.9%) in buccal tissue (Berko et al., 2014). Of these, *BCL2, RORA* (Nguyen et al., 2010), *DLL1* (Schroeder et al., 2016), *TAC1*(Aldinger et al., 2013), and *MGC3207* (*r* = 0.91) (Wong et al., 2014) were validated. Only one study did not find evidence for differential DNA methylation patterns between cases and controls of mixed age in blood tissue (Homs et al., 2016). However, the authors did report epigenetic differences in 700 CpG sites when each individual patient was compared to ten controls; 17 of these sites were replicated.

3.2.3 *Candidate Gene DNA Methylation*

The relation between DNA methylation and ASD was examined in 11 candidate gene DNA methylation studies collectively spanning 16 genes, 2 of which (*MeCP2* and *UBE3A)* were selected by multiple

authors. Seven publications investigated brain tissue while four examined peripheral tissue. Characteristics and findings of all candidate gene DNA methylation studies examining ASD can be found in Table 3.

Brain Tissue: Associations in *MeCP2* – a gene coding for a protein with a methyl-CpG binding domain ([dataset] Geer et al., 2010) - were examined by three studies in mixed-age samples but could not be replicated. Nagarajan et al. (2006) reported DNA hypermethylation in autistic patients in *MeCP2* region I in Brodmann's area 9 and in fusiform gyrus tissue. In a subsequent study, the same author showed no proof for statistically significant differences in region II of *MeCP2* in Brodmann's area 9 (Nagarajan et al., 2008). Ginsberg et al. (2012) reported no evidence for DNA methylation differences in *MeCP2* in Brodmann's area 19, occipital cortex and cerebellar hemispheric cortex, but they did not specify which genetic region was analyzed.

Other studies found differential DNA methylation distribution in autistic subjects of mixed age in the *RELN* promoter region within Brodmann's areas 41/42 and 22 ($M \pm SD$ = 29.55 ± 13.09 in cases, 5.70 ± 2.90 in controls) (Lintas et al., 2016) and higher DNA methylation in the *EN-2* gene within the cerebellum ($M \pm SD$ = 23±4% to 38±7% in cases, 10±2% to 15±5% in controls) (James et al., 2013). A replication was not attempted. No proof for significant differences was found in correspondence to *RORA, BCL2* and *OXTR* in Brodmann's area 19 and cerebellar hemispheric cortices (Ginsberg et al., 2012), *UBE3A* in Brodmann's area 19, cortices and cerebellum (Ginsberg et al., 2012; Jiang et al., 2004), *SNRPN* in cerebellum and cerebral cortex (Jiang et al., 2004), and *OCA2* in Brodmann's area 19 (Mitchell et al., 2012).

Peripheral Tissue: There was no attempt to replicate findings in candidate gene DNA methylation studies comparing autistic patients and controls in peripheral tissue. Hranilovic et al. (2016) found higher DNA methylation in autistic subjects of mixed age in a region of *HTR2A* – a gene encoding a receptor for serotonin ([dataset] Geer et al., 2010) - in the leukocytes of AG genotype carriers only. No evidence for significant differences was found in correspondence to *FOXK1* in blood (Atsem et al., 2016), *PEG1* and

COPG2 in leucocytes (Bonora et al., 2002) and *PEG13*, *KCNK9* and *TRAPPC9* in peripheral blood (Delgado et al., 2014).

3.2.4 *Histone Modifications*

The association between histone modifications and ASD was assessed by two studies in the brain tissue of mixed age populations (James et al., 2013; Sun et al., 2016). They analyzed different histone modifications (methylation and acetylation) on H3K27. James et al. (2013) reported lower trimethylation levels of H3K27 in the cerebellum of 11 autistic patients when compared to 11 controls. Consistently, Sun et al. (2016) found differential acetylation of H3K27 between 45 cases and 49 controls in the cerebellum, prefrontal and temporal cortices. No evidence for H3K4 to be differentially trymethylated was found in the cerebella of cases and controls (James et al., 2013). Further information regarding the studies examining histone modifications can be found in Table 4.

3.3 Attention-Deficit/Hyperactivity Disorder

The relation between epigenetic modifications and ADHD was investigated with the use of epigenome-wide and candidate gene approaches only.

3.3.1 *Epigenome-Wide DNA Methylation*

The relation between ADHD and epigenome-wide DNA methylation was examined by two studies (Walton et al., 2017; Wilmot et al., 2016) and in peripheral tissue only. Differential DNA methylation in Vasoactive Intestinal Peptide Receptor 2 (*VIPR2*) was found in two independent analyses carried out by Wilmot et al. (2016). In a discovery sample comprising 43 children with ADHD and 42 controls ranging from seven to 12 years, they identified differential DNA methylation in 95 genes, including *VIPR2* (β = -0.059). Subsequently, in a replication sample of 10 pairs of cases and controls, they confirmed DNA hypomethylation in one of the previously identified CpG sites in *VIPR2* (cg13444538) (β = -0.096).

Another study by Walton et al. (2017), using prospective data, reported contrasting epigenetic patterns in cord blood between 40 children with a chronic high vs 777 with a low trajectory of ADHD, in

the proximity of SKI (β = -0.2), EPX (β = -0.18), PEX2 (β = 0.17), ST3GAL3 (β = -0.17), FBXW5 (β = 0.17), ELF3 (β = 0.17), ZNF544 (β = 0.17). These epigenetic differences were no longer observable at age 7 in whole blood. Further information on the publications investigating epigenome-wide DNA methylation in ADHD can be found in Table 5.

3.3.2 *Candidate Gene DNA Methylation*

The relation between candidate gene DNA methylation patterns and ADHD was analyzed in three studies (Adriani et al., 2017; Van Mil et al., 2014; Xu et al., 2015) in peripheral tissues only. Associations between differential DNA methylation and ADHD in *SLC6A3/DAT1* – a gene encoding a dopamine transporter ([dataset] Geer et al., 2010) - were analyzed by two independent studies. Adriani et al. (2017) showed DNA hypomethylation of the *SLC6A3* gene in 30 cases compared to 15 controls ranging from six to 12 years in blood and buccal tissues. In contrast, Xu et al. (2015) did not observe differential DNA methylation patterns in *DAT1* between 50 children suffering from ADHD and 50 controls ranging from four to 12 years.

Associations between differential DNA methylation and ADHD in *DRD4* – a gene encoding a dopamine receptor ([dataset] Geer et al., 2010) - were found by two independent studies; however, the direction of the effect was inconsistent (Van Mil et al., 2014; Xu et al., 2015). Van Mil et al. (2014) reported DNA hypomethylation in *DRD4* in the cord blood of children with ADHD (β = -0.52). Xu et al. (2015) additionally showed DNA hypermethylation in individuals with ADHD in correspondence to *DRD4*.

Van Mil et al. (2014) also found hypomethylation of *5-HTT* in cord blood (β = -0.22). No evidence for significantly different epigenetic marks was identified in CpGs in, or near, *IGF2*, *H19*, *KCNQ1OT1*, *MTHFR* and *NR3C1* (Van Mil et al., 2014). More information on the studies examining candidate gene DNA methylation in ADHD can be found in Table 6.

4. DISCUSSION

The present work aimed to provide a comprehensive evaluation of the current evidence for the relation of differential epigenetic markers with ASD and ADHD. To our knowledge, this is the first systematic review examining epigenetic modifications in each neurodevelopmental disorder.

Of the 29 included publications, the majority focused on DNA methylation, examined autism and selected a cross-sectional design. Studies used different tissues and analytical approaches. These results provide preliminary support for an association between epigenetic alterations and neurodevelopmental disorders. Yet, the mixed findings, the small-sized samples, and the low comparability of publications prohibit definite conclusions.

4.1 Summary of key Findings

Of the small set of studies investigating global DNA methylation in relation to autism, some identified hypermethylation in cases while others found no evidence for differences between groups. Similarly, mixed results have been reported in the global DNA methylation patterns of individuals affected by neurodegenerative disorders such as Alzheimer's and Parkinson's, and other chronic diseases (Braun et al., 2016; Muka et al., 2016a; Muka et al., 2016b; Wen et al., 2016). Inconsistencies might stem from heterogeneity in *(i)* the tissues selected (e.g. blood vs brain tissue), *(ii)* the analytical and statistical methods used (e.g. differences in DNA methylation assessment methods, analytic routines and significance thresholds) and, *(iii)* the sample characteristics (e.g. age differences). Generally, hypothesis-free approaches (EWAS) did not yield consistent results although often the same array was employed. Nevertheless, the identification of differential DNA methylation in the proximity of three genes – *PRRT1, C11orf21/TSPAN32,* and *OR2L13* - in multiple independent analyses, is a promising preliminary finding. Mutations in the gene family of *PRRT1* are involved in neurological disorders such as familial infantile seizure (Ladd-Acosta et al., 2014). A similar role in normal/neurological phenotypes was described for the mice homolog *prrt1,* which is also implicated in central nervous system functioning ([dataset] Smith et al., 2018). The genomic area encompassing *C11orf21* and *TSPAN32* could constitute a bidirectional promoter,

hence a DNA region in which transcriptional processes of two genes are commenced in opposite orientations from a putative promoter. Given the important role of bidirectional promoters in gene regulation and in the production of both coding and non-coding RNA (Wei et al., 2011), we speculate that epigenetic alterations within *C11orf21/TSPAN32* might lead to pervasive effects in transcriptional regulation at a genome-wide level. The function of *C11orf21* is yet to be elucidated, although one study suggested its role in cytoplasmic localization (Zhu et al., 2000). In contrast, more information is available for *TSPAN32*, which is located within the tumor-suppressor gene region encompassing chromosome 11p15.5 ([dataset] Geer et al., 2010). DNA methylation alterations in *TSPAN32* were found to relate with Beckwith-Wiedemann syndrome ([dataset] Geer et al., 2010), a disease characterized by genetic and epigenetic dysregulation ([dataset] Shuman et al., 2016; Weksberg, 2010). The increased incidence of autism among individuals suffering from Beckwith-Wiedemann syndrome ([dataset] Shuman et al., 2016; Betancur, 2011) suggests that *TSPAN32* DNA methylation status might play a role in both disorders. Moreover, mutations in another member of the tetraspanin family, *TSPAN8*, were described in other psychiatric conditions, namely in schizophrenia and bipolar disorder (Scholz et al., 2010). *OR2L13* is responsible for the initialization of the neuronal response to odorants (Berko et al., 2014). Mice studies have also observed involvement in olfactory receptor activity in the homologs of *OR2L13*, *olfr166* and *olfr168* ([dataset] Smith et al., 2018). Olfactory dysfunctions have been reported in autistic patients and were found to correlate with the social impairment component of the disorder (Bennetto et al., 2007; Hilton et al., 2010). DNA methylation differences in *OR2L13* between cases and controls were not found in monozygotic twins discordant for the disorder (Wong et al., 2014), indicating the possibility of an influence of genetic variants on these epigenetic markers. As evident through a mQTL database search (Gaunt et al., 2016), the CpG of interest (cg20507276) was indeed related to numerous single nucleotide polymorphisms. Yet results should be interpreted with caution because of numerous caveats in the literature, such as the very small sample size employed and the use of platforms with lower CpG coverage (Infinium 27k array). A number of shared biological pathways of relevance to neurodevelopment were

reported by independent EWAS, including synaptic and neuronal processes (Nardone et al., 2017; Nguyen et al., 2010), immune response processes (Nardone et al., 2017; Nardone et al., 2014; Nguyen et al., 2010), brain development (Nguyen et al., 2010) and cellular differentiation (Table 2). Candidate gene studies of autism identified DNA methylation differences in multiple genes; however, the absence of replication implies that the field lacks credible data to assess how DNA methylation of specific *a priori* genes associates with autism. Histone modifications (trimethylation and acetylation) in association with ASD were consistently found in H3K27 in the cerebellum and cortices of autistic patients. Yet, a role of these variations in autism can only be inferred very cautiously due to the high reversibility and complicated mechanisms that histone modifications entail.

Associations of epigenetic patterns with ADHD were explored in a few studies only, which focused on site-specific DNA methylation. Overall, epigenome-wide approaches to DNA methylation analysis provided inconsistent findings. Nevertheless, *VIPR2* was recognized as hypomethylated in two independent samples by Wilmot et al. (2016). Duplications and SNPs in *VIPR2* seem to be implicated in schizophrenia and mood disorders, respectively. *Vipr2* was also found to be involved in hypo-activity in mice models ([dataset] Smith et al., 2018). Based on this finding and on their research, Wilmot et al. (2016) speculated the possibility of a relation between human hyperactivity symptoms and decreased DNA methylation in *VIPR2*. Further investigations are needed. Interestingly, both *VIPR2* (for ADHD) and *OR2LI3* (for ASD) are involved in the signaling by G-protein-coupled-receptors (GPCR) pathway ([dataset] Weizmann Institute of Science, c2018), which includes genes responsive to stimuli external to the cell, including odors and neurotransmitters. This might indicate that epigenetic dysregulation of the signaling by GPCR pathway could be characteristic of both neurodevelopmental disorders; however, it should be noted that only two of the 2’601 genes included in this network were observed as differentially methylated. Further pathways involved in ADHD shared by non-replicated sites/regions include peroxisomal processes and transcriptional activity (Table 5) (Wilmot et al., 2016). Candidate gene DNA

methylation studies of ADHD could not find consistent evidence of differential DNA methylation between cases and controls or did not attempt to replicate their results.

4.2 Current Challenges in the Field and Recommendations for Future Research

When interpreting the reported findings, multiple methodological issues should be acknowledged. First, the selected *DNA methylation assessment method* (global, candidate gene and genome-wide DNA methylation analysis) might explain reported inconsistencies (e.g. direction of DNA methylation differences – hypo, hyper – and lack of converging findings in epigenome-wide and candidate gene studies). Global DNA methylation might not be a sufficiently fine-grained method since epigenetic modifications in psychiatric phenotypes are likely to be subtle and site-specific compared to disorders characterized by large-scale genomic instability. Although epigenome-wide and candidate gene approaches permit a more detailed assessment of DNA methylation, these two methods diverge on several aspects. With the setback of higher statistical burden and costs, epigenome-wide analysis permits a wider coverage of the genome and the discovery of differentially methylated genes, which could otherwise be disregarded. A hypothesis-free approach thus appears the most appropriate when knowledge on the epigenetics of the disorder is limited, as in ASD and ADHD. Yet, epigenome-wide studies require more stringent guidelines before we can successfully identify true and consistent effects (Heijmans and Mill, 2012). Overall, we are confident that once EWAS are more standardized and well-powered, it will be possible to meta-analyze findings and to detect consistent alterations in DNA methylation.

Second, the selection of *statistical analyses* likely influenced the detection of differentially methylated patterns. The included studies mainly analyzed differentially methylated positions (site-by-site analysis) or regions (regional change analysis). The former consider the association of each differentially methylated site with the disorder of interest, while the latter provide a proportion of the methylated cytosines in a specific area of the gene (Mill and Heijmans, 2013). Currently, there is no consensus on the most appropriate statistical analysis for epigenetic studies. Combination of approaches would enable the

identification of single abnormally methylated CpGs (site-by-site analysis), as well as the clustering of correlated sites (regional analysis or other reduction-based strategies) which are likely to be functionally relevant. This was performed in few of the reviewed studies (Aldinger et al., 2013; Homs et al., 2016; Nardone et al., 2014), which exemplify the current developments in the field.

Most of the selected publications measured differential DNA methylation patterns based on bisulfite-treated DNA, which involves the deamination of non-methylated cytosines to uracil while leaving unaltered the methylated cytosines. A major setback of bisulfite conversion is the inability to distinguish between 5-mC and 5-hydroxymethylcytosine (5-hmC), another form of epigenetic modification involving the oxidation of 5-methylcytosine (Huang et al., 2010). Profiling 5-hmC separately could be insightful for ASD and ADHD research, given its role in neurodevelopment and disease. For instance, together with TET proteins, 5-hydroxymethylcytosine is considered to be involved in the DNA demethylation of 5-mC occurring during epigenetic reprogramming in the developing germline or in the early embryonic phase (Hill et al., 2014). In line with this, developmental regulation of 5-hmC quantities, with an increase from early postnatal stage to adulthood, has been reported in mice brain tissue (Szulwach et al., 2011). Of the included studies, none distinguished 5-hmC from 5-mC, indicating that the reported findings might not accurately portray the DNA methylation patterns of the participants, especially in brain tissue where 5-hmC is enriched. The implementation of methods enabling the distinction between 5-mC and 5-hmC, such as oxidative bisulfite sequencing (Booth et al., 2012), could enable advancements in the understanding of the epigenetic processes involved in neurodevelopmental and related disorders.

A key issue in epigenetic research and especially in the included publications is the use of *small sample sizes*. Although there are no general guidelines, Tsai and Bell (2015) estimated the minimum number of participants necessary to reach 80% power according to multiple factors such as the mean DNA methylation difference present between affected and unaffected individuals. In particular, they reported that EWAS should include at least 54 case-control pairs to have adequate power to detect a 15% mean DNA methylation difference at a genome-wide significance. With the exception of a couple of studies

(Homs et al., 2016; Wang et al., 2014), none of the included publications met this criterion indicating that most epigenome-wide analyses likely did not have the power to detect true DNA methylation differences. It is thus of utmost importance to consider the gathered results as preliminary and to interpret them with caution. It should also be noted that since mean DNA methylation differences between cases and controls at single CpG sites are likely to be small in effect size, much larger samples should be employed.

The included publications were mostly comparing cases and controls cross-sectionally, meaning that causal inferences could not be drawn due to the presence of the disorder during assessment. Contrarily, a prospective *design*, which allows for measuring epigenetic patterns pre-symptom onset, could elucidate the direction of the epigenetics-disorder relation. Monozygotic-twin discordant studies are another substantial resource as they control for the influence of genetic variants and display DNA methylation changes arising from discordant environmental exposures. The results stemming from both monozygotic-twin discordant and longitudinal designs should be integrated to identify the direction of the epigenetics – disorder relation (longitudinal design), as well as the influence of genetic variants on epigenetic markers (twin-discordant study). The use of repeated measurements in longitudinal studies would provide further insight, as exemplified by Walton et al. (2017), whose results varied with age of the children.

Regardless of the design, findings can be hampered by *confounding variables* influencing the association between DNA methylation and symptomatology of the disorder and causing inflated and inaccurate results. Although the majority of the included publications corrected for potential third variables, among which sex and age were the most common, adjustments for technical covariates such as batch effects and cell type were performed by a few studies only (e.g. Berko et al., 2014; Nardone et al., 2017).

The included publications mainly examined brain and blood. Because of *epigenetic tissue specificity* (i.e. each tissue has its specific epigenetic patterns), peripheral tissues may not be appropriate surrogates to estimate alterations in the brain of individuals affected by neurodevelopmental disorders.

Yet, modifications of early development might present higher inter-tissue concordance (Heijmans and Mill, 2012). The brain may be the most suitable source for the identification of disease biomarkers (Bakulski et al., 2016), but its analysis can be problematic and highly impractical due to limited availability of post-mortem data, the impossibility to infer causality, and degeneration of the genetic material (Wen et al., 2016). In addition, as epigenetic marks can change across the lifetime (Bell and Spector, 2011), modifications measured after patients' death may not be representative of the ones present during disorder onset or course. Peripheral blood is easier to access and can be collected at any age, it enables analysis of larger samples and can be examined longitudinally (Bakulski et al., 2016). Interestingly, Smith et al. (2015) suggested saliva as a more appropriate peripheral tissue than blood, due to its greater similarity to brain tissue. Importantly, since we are unaware of whether neurodevelopmental disorders depend on DNA methylation variations in the brain only or in multiple tissues, we suggest the analysis of different tissue types. When this approach is not feasible, consulting online resources showing correlations between peripheral tissues and brain is advised. Moreover, focusing on the use and development of non-invasive techniques for *in vivo* brain analysis, such as brain imaging, is of importance (Rakyan et al., 2011).

Epigenetic patterns not only vary across tissues, but also within tissues as these contain a mixture of cell types such as leucocytes and red blood cells in whole blood or neurons and glial cells in brain. Failure to account for such heterogeneity can severely confound results. For instance, if cases and controls diverge in their cell-type proportions, observed epigenetic differences might reflect cell-type composition rather than the phenotype of interest (Bock, 2012; Rakyan et al., 2011). The methods commonly employed to address cellular heterogeneity include cell sorting, reference-based (e.g. Houseman's constrained projection) and reference-free methods (e.g. RefFreeEWAS). Cell sorting provides the exact proportion of different cell types in a specific sample but is more costly and labor-intensive than the alternatives. Reference-based and reference-free methods, estimating cell-type composition with algorithms (Teschendorff and Zheng, 2017), provide a more feasible yet less accurate option. Of the reviewed studies,

only a minority corrected for cell-type heterogeneity. Most publications used reference-based methods, a few studies selected histologically identical cells, purified cells or reference-free methods, and two employed cell sorting (Nardone et al., 2017; Wong et al., 2014). Given the importance of accounting for cellular heterogeneity, Birney et al. (2016) suggested analyzing purified or histologically identical cells together with using any mean available to quantify cell-type proportions.

In the reviewed studies, epigenetic findings tended to be solely based on *statistical significance*. It is however unclear to what extent statistically significant CpGs/regions reflect a functional effect (i.e. variation in the phenotype). In order to solve this issue, Barker et al. (2017) proposed the integration of DNA methylation data with other *omics* (e.g. proteomics), the use of animal models and of *in vitro* experiments. Several studies conducted gene expression or gene set enrichment analyses and could thus bridge the gap between statistical and functional significance (e.g. Aldinger et al., 2013; Ginsberg et al., 2012).

The limited understanding of the *role of epigenetics in mental health disorders* constitutes another key challenge in this field. Epigenetic modifications could exert a causal role in disorder etiology by mediating or modifying genetic or environmental risk or by representing the biological mechanism for gene-environment interactions (Ladd-Acosta and Fallin, 2016). Conversely, if not pathways to disorder onset, epigenetic alterations could be biomarkers of exposure to environmental factors or to the disorder itself. If epigenetics mediates the relation between environment and disorder, scientists might be able to help reverse psychiatric conditions by altering nutrition, behavior, social factors, and with the use of drugs (Rutten and Mill, 2009). On the other hand, improvements in the accuracy of diagnoses and of illness detection could be achieved in case epigenetic modifications are biomarkers of disease (Ladd-Acosta and Fallin, 2016). Characterizing the role of epigenetics with respect to disorder, environment, and genotype would greatly contribute to moving the field forward, enabling appropriate interpretation of epigenetic findings and informing potential translational applications for public health.

In conclusion, current evidence suggests the possibility of an association between epigenetic modifications and neurodevelopmental disorders. Yet, with very few exceptions, the results were either conflicting or not independently replicated. The available research is limited and hampered by small samples, suboptimal designs and heterogeneity in approaches, analyses and tissues. While scientists are still far from understanding the causal, diagnostic or prognostic role of epigenetics in neurodevelopmental disorders, this field is exciting due to its rapid development and advances. Longitudinal and genetically-sensitive designs, increased samples sizes, adjustments for appropriate confounders and integration of *omics*-data will permit the delineation of the role of this promising field in ASD and ADHD. Careful investigations of epigenetic processes in relation to these and other neurodevelopmental disorders are highly recommended.

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Figure captions:

Figure 1. PRISMA flow diagram of the studies included in this systematic review.

Of the 2174 retrieved articles, after deduplication, we screened the title and/or abstract of 2169 publications and identified 86 potentially relevant ones. Based on a full-text assessment of their eligibility, 29 publications were included in this systematic review. *Abbreviations:* n=number.

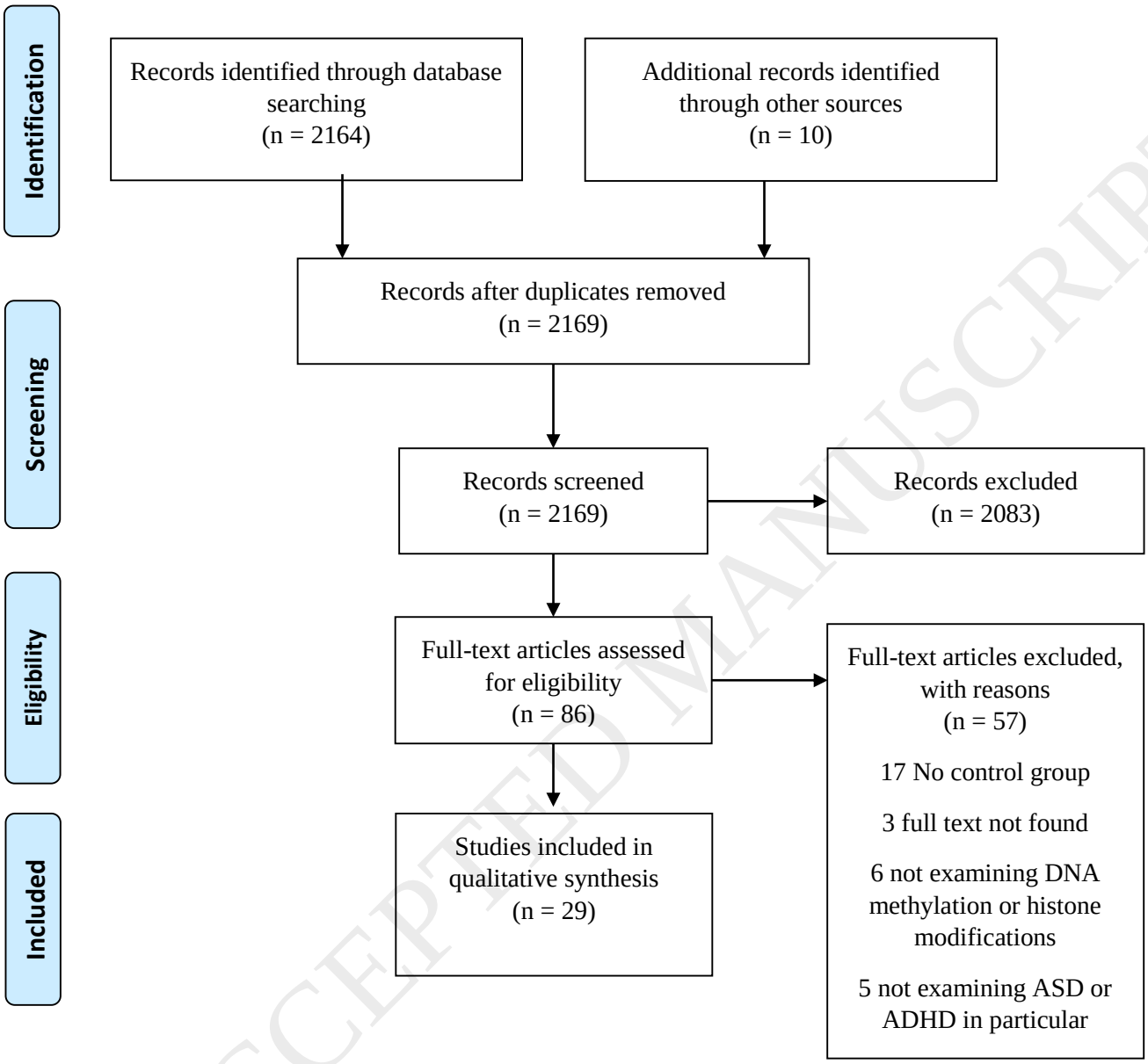

Table 1.

Global DNA methylation and Autism Spectrum Disorder.

Author	Cases /Controls	Sample Characteristics	Tissue	Adjustments/Matching	Method	Findings	Significance Level
Brain tissue							
James et al. (2013)	13/13 from 2 samples	USA 4-30 y. M and F	CB	Matched by age, sex, race, PMI and cause of death.	LC-MS/MS	Hypermethylation (5.9±1.2 in ASD patients, 4.7±1.1 in controls).	$p < 0.05$
Mitchell et al. (2012)	12/12	USA 4-61 y. M and F	BA19	Sex, birth period, brain region /age and sex.	LINE-1	No significant differences.	$p < 0.05$
Peripheral tissue							
Schroeder et al. (2016)	24/23	USA Birth to 36m. M and F	Placenta, CBL and non-specified	Sex, sequencing run number, order of sequencing, average sequencing coverage / sex and birth year.	MethylC-sequencing	No significant differences.	FDR < 0.05
Tsang et al. (2016)	280/236	China M_{ASD} = 4.7 y. $M_{controls}$=5.3 y. M and F	Leukocytes	Matched by age.	HpaII/MspI	Hypermethylation ($\Delta\beta$ = 54.35±21.37) in autistic subjects.	$p < 0.05$
Wong et al. (2014)	66/44	UK Birth to 15y. sex NA	WB	Adjusted for blood cell count.	LUMA	No significant differences (M = 65.1% in autistic twin, M = 65.9% in unaffected twin).	$p < 0.82$ (based on observed p-value)

ASD = Autism Spectrum Disorder; BA Brodmann's Area; CB = cerebellum; CBL = cord blood; F = females; FDR = false discovery rate; M = males; m. = months; NA = not available; PMI = *post-mortem* interval; WB = whole blood; y. = years.

Table 2.

Epigenome-wide DNA methylation and Autism Spectrum Disorder.

Author	Cases /Controls	Sample Characteristics	Tissue	Statistical Analysis	Platform	Replication Validation	Adjustments /Matching	Findings	Significance Level
Brain tissue									
Feinberg et al. (2015)	19/21	NA	FC, TC, CB	DMR	Infinium 450k array	None	None	Differential DNA methylation in *SNORD* family genes in CB, in 12 regions in FC and 10 regions in TC.	$p < 0.05$
Ginsberg et al. (2012)	17/17 from 2 samples	USA, UK, Canada and unknown 1-60 y. M	BA19 and cerebellar hemispheric cortex.	DMP	Infinium 27k array	None	Region and age / age and sex.	No significant differences.	FDR ≤ 0.05 and FDR ≤ 0.25
Ladd-Acosta et al. (2014)	20/21 from 3 samples	USA NA	TC, PFC, CB	DMR	Infinium 450k array	Replication, 25/26	Sex, cell type / age, sex and PMI	DNA hypomethylation in cases in *PRRT1* (ΔM = -7.8%.), *C11orf21/TSPAN32* (ΔM = -6.6%) and DNA hypermethylation in *ZFP57* (ΔM = 13.9%) in cases in TC. DNA hypermethylation in *SDHAP3* in cases in cerebellum (ΔM = 15.8%). Differential DNA methylation replicated in the same regions encompassing *PRRT1* and *C11orf21/TSPAN32*. **NB:** Replication analyses were conducted in a different brain tissue of the same individuals in	DMR: FWER ≤ 0.1 Replication: $p <$ 0.05

Author	Cases /Controls	Sample Characteristics	Tissue	Statistical Analysis	Platform	Replication Validation	Adjustments /Matching	Findings	Significance Level
								the discovery cohort.	
Nardone et al. (2017)	15/16 from 2 samples	UK, USA 17-68 y. M	PFC, BA10, BA9, BA8	DMR DMP	Infinium 450k array	Validation with NGBS	Technical and biological covariates / age, brain mass and PMI	No significant differential DNA methylation in DMP analysis. Differential DNA methylation in 58 regions, spanning *GABBR1, ABAT, Mir124-1, Mir124-2* in DMR analysis DNA hypomethylation in *GABBR1* and *Mir124-2* and DNA hypermethylation in *FAM124B* and *InNEAT1* in cases validated. Pathways: synaptic and neuronal processes and immune response processes.	DMP and DMR: FDR < 0.05 Validation: $p <$ 0.05
Nardone et al. (2014)	24/23 from 2 samples	UK, USA 16-51 y. M and F	BA10, BA24	DMP	Infinium 450k array	Validation with pyrosequencing	Adjusted for sex and batch effects	Differential DNA methylation in 5329 CpGs in BA10 and 10745 in BA24, including *HDAC4, PRRT1, C11orf21/TSPAN32, C1qA, IRF8, CTSZ.* DNA hypomethylation of *C11orf21* ($\Delta\beta$ = 0.39 to -4.93) and *C1qA* ($\Delta\beta$ = -3.45), *IRF8* ($\Delta\beta$ = -3.42), *CTSZ* ($\Delta\beta$ = -2.13 to -3.39) in BA10 in cases validated. Pathways: immune responses, neuron-neuron synaptic transmission.	DMP: FDR < 0.05 Validation: $p <$ 0.06 (based on observed *p*-values)
Peripheral tissue									

Author	Cases /Controls	Sample Characteristics	Tissue	Statistical Analysis	Platform	Replication Validation	Adjustments /Matching	Findings	Significance Level
Aldinger et al. (2013)	4/6	USA Age NA F	LCL	DMP and grouping of CpG sites	Infinium 27k array	Replication in 13/13. Validation with MassArray EpiTYPER.	Matched by age	Differential methylation in 261 probes ($\|\Delta\beta\| > 0.15$), including probes in *PTCD2*, *TAC1*, *HOXA11*, *TCN1*, *ETNK2*. Results not replicated. Differential DNA methylation in *PTCD2*, *FLJ44881*, *SPAG7*, *TAC1* in the pooled analysis. *TAC1* validated. .	DMP: $p < 0.01$, FDR < 0.10 Replication and pooled: $p < 0.0005$ Validation: $p < 0.005$
Berko et al. (2014)	47/48	USA 1-28 y. M and F	Buccal tissue	DMR	Infinium 450k array	Validation with NGBS	Adjustment for sex, age, ancestry, SNPs, technical variables, ASD status, microarray-based variables and batch effects	Differential DNA methylation in 13 genes: *OR2L13*(β = -8.0%), *PAX8* (β = -7.5%), *GPC1* (β = -8.6%), *ADRA2C* (β = -11.9%), *FAM134B* (β = -7.8%), *CREB5* (β = 7.6%), *NOS1* (β = 8.6%), *MAPK8IP3* (β = 9%), *HOOK2* (β = -7.9%), *NRG2* (β = 7.6%), *KCNQ5* (β = 7.7%), *ZG16B* (β = 10.1%), *LOC643802* (β = -8.9%). *OR2L13* validated as hypomethylated.	DMP:1-$\log_{10}$ (based on observed values for *FAM134B*, *ORL13*) or unclear (for other genes). Validation: $p < 0.05$
Homs et al. (2016)	53/757 from 4 samples	Spain, USA and other Europeans Age 2-101 M and F	PB, CBL, WB	DMP, DMR and individual analysis (each single case compared to 10 controls)	Infinium 450k array	Replication in 415/405. Validation with Sanger BS and pyrosequencing.	Matched by age in one sample.	No significant differences in DMP analysis. Differential DNA methylation in DMR analysis in five regions in four patients only. Differential DNA methylation in 700 CpGs in the individual analysis due to rare genetic variants. Differential DNA methylation in individual analysis replicated in	DMP: $q \leq 0.01$. DMR: $p < 0.01$ Individual analysis:$p < 0.01$, f < 0.005

Author	Cases /Controls	Sample Characteristics	Tissue	Statistical Analysis	Platform	Replication Validation	Adjustments /Matching	Findings	Significance Level
								17/700 CpGs. Differential DNA methylation in DMR validated. Pathways: AMP-activated protein kinase signaling.	
Nguyen et al. (2010)	5/9	USA 2-19y M	LCL	DMP	MIRA	Validation with MSP or Sanger BS	None	Differential DNA methylation in 73 CpG islands between discordant monozygotic twins. Differential DNA methylation in 201 CpG islands when comparing both twins to unaffected siblings. DNA hypermethylation in *BCL-2* (*M* = ~25% in ASD, *M* = 9.5% in controls) and *RORA* in cases. Results were validated. Pathways: inflammation and apoptosis, brain morphogenesis, cellular differentiation, growth rate, myelination, cytokine production, synaptic regulation, steroid biosynthesis, learning.	DMP: log2 - 0.938 (based on observed values) Validation: not reported.
Schroeder et al. (2016)	24/23	USA Birth to 36m. M and F	Placenta	Grouping of CpG sites	WGBS	Validation with pyrosequencing	Sequencing run, order, coverage, child ethnicity, sex / sex and birth-year.	DNA hypermethylation near *DLL1* and *LOC15444* in cases. Results were validated.	Grouping of CpG sites: FDR ≤ 0.05 Validation: $p <$ 0.05.
Wang et al. (2014)	5/5	China 3-12y. M and F	Venous B	DMR (promoters and CpG islands)	MeDIP	Replication, 131/131 Validation with Sanger BS	Matched by age and sex.	Differential DNA methylation in 228 gene promoters and 247 CpG islands when comparing 5 cases and 5 controls. DNA hypermethylation in *ENO2* promoter replicated in 15% of the replication sample (*M* = 39.1% in autistic patients, *M* = 18.81% in controls).	DMR: $p < 0.05$ Replication: $p <$ 0.01 Validation: $p <$ 0.01

Author	Cases /Controls	Sample Characteristics	Tissue	Statistical Analysis	Platform	Replication Validation	Adjustments /Matching	Findings	Significance Level
Wong et al. (2014)	66/44	UK Birth to 15y. M and F	WB	DMP (one for discordant twins, one for cases vs. controls)	Infinium 27k array	Validation with pyrosequencing	Adjusted for cell count.	DNA hypomethylation in the promoter of *PIK3C3* in affected twins ($\Delta\beta$ = -0.04) in DMR. DNA hypomethylation in *NFYC* in affected twin in DMP analysis. Differential DNA methylation in *MGC3207*($\Delta\beta$ = -0.24), *OR2L13*($\Delta\beta$ = 0.18) and *C14orf152(*$\Delta\beta$ = -0.16) between cases and controls. *OR2L13* (r = 0.86) and *MGC3207* (r = 0.91) validated.	DMP (twins): $p <$ 0.018 (based on observed p-values for top 50 hits) DMP (cases-controls): $p <$ 0.00062 (based on observed p-values for top 50 hits) Validation: not reported.

ASD = Autism Spectrum Disorder; B = blood; BA = Brodmann's Area; CB = cerebellum; CBL = cord blood; DMP = differentially methylated positions; DMR = differentially methylated regions; F = females; FC = frontal cortex; FDR = false discovery rate; FWER = familywise error rate; LCL = lymphoblastoid cell lines; M = males; NA = not available; PB = peripheral blood; PFC = prefrontal cortex; PMI = post-mortem interval; = TC = temporal cortex; WB = whole blood; WGCNA = weighted gene co-expression network analysis; y. = years.

Table 3.

Candidate gene DNA methylation and Autism Spectrum Disorder.

Author	Cases /Controls	Sample Characteristics	Tissue	Gene	Statistical Analysis	Platform	Adjustments /Matching	Findings	Significance Level
Brain tissue									
Ginsberg et al. (2012)	>7/>7	USA, UK, Canada and unknown 1-60 y. M	BA19 and cerebellar hemispheric cortex.	*RORA, BCL2, UBE3A, MECP2, OXTR, CEBPD*	DMP	Pyrosequencing	Matched by age and sex.	No significant differences.	FWER ≤ 0.05
James et al. (2013)	13/13 from 2 samples	USA 4-30 y. M and F	CB	*EN-2* (promoter)	DMP	Restriction enzymes	Matched by age, sex, PMI, cause of death and race.	DNA hypermethylation in autistic patients in all restriction enzymes used (McrBC-PCR analysis:23±4% of DNA methylation in ASD, 10±2% of DNA methylation in controls); (MSR-PCR :38±7% in ASD, 15±5% in controls).	$p < 0.05$
Jiang et al. (2004)	17/60 from 3 samples	NA	CB, CC	*SNRPN* 5'-end, *UBE3A* (3'and 5'-end)	DMR	Restriction enzymes	None	Differential DNA methylation in one participant in *UBE3A.* **NB:** This was the only candidate gene DNA methylation study performing validation (Sanger BS).	Not reported
Lintas et al. (2016)	6/6 from 3 samples	USA 16-30 y. 10M and 2F	BA41/42, BA22	*RELN* (promoter)	DMR	Sanger BS	Matched by age, sex and PMI.	Differential DNA methylation distribution. Hypermethylation in the 5' portion in autistic subjects (29.55±13.09 in cases, 5.70±2.90 in controls).	$p < 0.05$
Mitchell et al. (2012)	12/12	USA 4-61 y. M and F	BA19	*OCA2* (promoter)	DMP	Pyrosequencing	Sex, birth period, brain region/ age and sex	No significant differences.	$p < 0.05$

Author	Cases /Controls	Sample Characteristics	Tissue	Gene	Statistical Analysis	Platform	Adjustments /Matching	Findings	Significance Level
Nagarajan et al. (2008)	9/9 from 4 samples	USA 2-21 y. M and F	BA9	*MECP2* (promoter region II)	DMR	Sanger BS	Matched by age, sex and geographical area.	No significant differences.	$p < 0.07$ (based on observed *p*-value)
Nagarajan et al. (2006)	20/27 from 4 samples	USA 76days – 57 y. 32M	BA9, FG	*MECP2* (promoter region I)	DMR	Sanger BS	None	DNA hypermethylation in autistic subjects. **NB:** Site 3 of region I is the only site of *MeCP2* showing a statistically significant higher DNA methylation in cases than controls.	$p < 0.05$.
Peripheral tissue									
Atsem et al. (2016)	74/41 from 3 samples	France, Germany 2-20y. M	B	*FOXK1*	DMR	NGBS	Age/age and sex	No significant differences.	Not reported.
Bonora et al. (2002)	46/10	Consortium NA	L	*PEG1/MEST, COPG2* (CpG islands)	DMR	Restriction enzymes	None	No significant differences.	Not reported.
Delgado et al. (2014)	86/48	Spain Age and sex NA	PB	*PEG13, KCNK9, TRAPPC9*	DMR	Pyrosequencing	None	No significant differences.	Not reported
Hranilovic et al. (2016)	90/66	Croatia 4-60 y. M and F	L	*HTR2A* (promoter)	DMR	Sanger BS	Age, sex and genotype/ age and genotype	DNA hypermethylation in the AG subgroup of autistic patients. No significant differences for the AA and GG subgroups.	Adjusted $p \leq 0.05$

ASD = Autism Spectrum Disorder; B = blood; BA = Brodmann's Area; CB = cerebellum; CC = cerebral cortex; DMP = differentially methylated positions; DMR = differentially methylated regions; F = females; FG = fusiform gyrus; FWER = familywise error rate; L = leucocytes; M = males; NA = not available; NB = *nota bene;* PB = peripheral blood; PMI = *post-mortem* interval; RBC = red blood cells; y. = years.

Table 4.

Histone modifications and Autism Spectrum Disorder.

Author	Cases/Controls	Sample Characteristics	Tissue	Histone and Lysine	Adjustments/Matching	Findings	Significance Level
James et al. (2013)	11/11	USA 4-30y. M and F	CB	H3K27 H3K4	Matched by age, sex, race, cause of death and PMI.	H3K27 trimethylation levels decreased in ASD individuals. No significant trymethylation differences in H3K4.	$p < 0.05$
Sun et al. (2016)	45/49	Consortium 10-81 y. M and F	PFC, TC, CB	H3K27	Adjusted for sex, age, proportion of neurons, multiple technical covariates.	Differential acetylation in cortices and cerebellum.	$q \leq 0.05$

ASD = Autism Spectrum Disorder; CB = cerebellum; F = females; H = histone; K = lysine; M = males; PFC = prefrontal cortex; PMI = *post-mortem* interval; TC = temporal cortex; y. = years.

Table 5.

Epigenome-wide DNA methylation and Attention-Deficit/Hyperactivity Disorder.

Author	Cases /Controls	Sample Characteristics	Tissue	Statistical Analyses	Platform	Replication Validation	Adjustments/ Matching	Findings	Significance Level
Walton et al. (2017)	Birth: 40/777 7y.: 50/842	UK Birth to 15 y. M and F	CBL and other peripheral tissues	DMP	Infinium 450k array	None	Adjusted for sex, chip and cell type, batch effects.	Differential DNA methylation in *SKI* (β = -0.2), *EPX* (β = -0.18), *PEX2* (β = 0.17), *ST3GAL3* (β = -0.17), *FBXW5* (β = 0.17), *ELF3* (β = 0.17), *ZNF544* (β = 0.17) at birth. No significant differences at age 7. **NB** This is the only study employing multiple measurements of DNA methylation.	q < 0.05
Wilmot et al. (2016)	43/42	USA 7-11.8 y. M	Saliva	DMP	Infinium 450k array	Replication in 10/10 Validation with NGBS	Medication status, age, race and correlation among genes/age	Differential DNA methylation in 95 genes including *VIPR2* (probe cg13444538) (β = -0.059). DNA hypomethylation in *VIPR2* replicated (β = -0.096) in ADHD subjects. DNA hypomethylation of 43 CpG sites validated in ADHD patients. Pathways: peroxisomal processes and transcriptional activity.	Discovery: Δβ > 2%, $p < 0.05$ Replication and validation: FDR < 0.05

ADHD = Attention-Deficit/Hyperactivity Disorder; CBL = cord blood; DMP = differentially methylated positions; F = females; FDR = false discovery rate; M = males; NB = *nota bene*; y.= years.

Table 6.

Candidate gene DNA methylation and Attention-Deficit/Hyperactivity Disorder.

Author	Cases /Controls	Sample Characteristics	Tissue	Gene	Statistical Analysis	Platform	Adjustments /Matching	Findings	Significance Level
Adriani et al. (2017)	30/15	Italy 6-14 y. M and F	B and buccal tissues	*SLC6A3* (6 CpG sites)	DMP	Pyrosequencing	None	DNA hypomethylation in all six sites in all ADHD patients. CpGs at position M1 significantly correlated with index scores for ADHD severity.	$p<0.05$ or $0.10 < p < 0.05$
Van Mil et al. (2014)	92/334	The Netherlands Birth to 6 y. M and F	CBL	*5-HTT, DRD4, IGF2DMR, H19, KCNQ1OT1, MTHFR, NR3C1*	DMR (CpG islands)	MassARRAY EpiTYPER	Adjusted for maternal education, age, prenatal smoking, parity, use of alcohol, folic acid supplement use, child national origin, height and weight, BMI, batch effects and psychological problems, child sex, Apgar score (one minute after birth), birth weight, mode of delivery, gestational age at birth, genetic ancestry, at birth, psychological problems comorbid with ADHD and genotype.	DNA hypomethylation in *5-HTT* region B (β=-0.22) and in the *DRD4* region (β=-0.52) when higher presence of symptoms in ADHD.	$p < 0.003$ (based on observed *p*-values)
Xu et al. (2015)	50/50	China 4-12 y. M and F	B	*DRD4*, *DAT1*(promoters)	DMP and DMR (CpG island)	Sanger BS	Child and parental characteristics/ age, sex and SES.	DNA hypermethylation in site 1 of *DRD4*. No significant differences in *DAT1*.	$p < 0.05$

ADHD=Attention-Deficit/Hyperactivity Disorder; B=blood; BMI= body mass index; CBL=cord blood; DMP=differentially methylated positions; DMR=differentially methylated regions; F=females; M=males; SES=socio-economic status; y=year